Cytotoxic Effects of Treosulfan on Prostate Cancer Cell Lines

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Abstract. Objective: Despite various therapeutic options in metastatic prostate cancer, the lack of a curative approach motivates further investigations. Treosulfan is an alkylating agent that has proven its indication in the treatment of e.g. ovarian carcinoma. This study focused on the objective of evaluating the effect of in vitro intoxication of human prostate carcinoma cell lines with treosulfan. Materials and Methods: Human prostate cancer cell lines LNCaP, DU145 and PC3 were treated with treosulfan concentrations from 0.5-500 μM for up to six days. Analysis of cell viability was performed using colorimetric WST-1 assay. Control data were obtained from identical cell lines cultivated without treosulfan. Results: Incubation with treosulfan inhibited cell viability and led to cell death in all cell lines in a dose- and time-dependent manner. After one day, viability of LNCaP, DU145 and PC3 cells was constantly reduced with a dose rate of at least 10 μM (p<0.001), 10 μM (p<0.0001) and 100 μM (p<0.0001) treosulfan, respectively. Minimum dose rates leading to death of nearly all LNCaP, DU145 and PC3 cells were 250 μM, 100 μM and 200 μM treosulfan, respectively. Conclusion: The results demonstrate a sensitivity of prostate carcinoma cells to the cytotoxic activity of treosulfan. Therefore, treosulfan might be a promising compound for novel treatment protocols for prostate cancer.

Prostate cancer is one of the most common malignancies found in men with a reported yearly incidence rate of 2.6 million in Europe comprising 11% of all male cancers in Europe (1). Over the past decade due to increasing sensitivity of serum prostate-specific antigen (PSA) monitoring assays, patients with prostate cancer have been diagnosed and subsequently have been treated with local treatments such as surgery and radiation therapy. Up to 40% of men treated with curative intention by local radiation therapy or surgery will experience an asymptomatic biochemical PSA relapse predating the actual onset of imagable metastatic disease for 3 to 5 years (2).

Once distant metastases in hormone-refractory disease develop, the prognosis is poor, with a median survival of only 16 to 18 months despite the introduction of chemotherapy (3). Data of clinical trials with docetaxel combined with estramustine and corticosteroids implied a survival improvement, with median survivals reported to be 14 to 23 months. Based on the encouraging data of two multicenter, randomised international trials comparing docetaxel+ estramustine with prednisone+mitoxantrone (3, 4), new regimens combining docetaxel with calcitriol, estramustine plus bevacizumab and thalidomide are being investigated in phase II and III trials.

From our own experience with docetaxel in a combined regimen, the level of prostate-specific antigen rises within approximately two to six months after completion of the treatment period after an initial drop under chemotherapy without exception (5). Novel modalities like anti-sense oligonucleotides (6), the proteosome and endothelin receptor antagonists (7), angiogenesis inhibitors (8), tumor vaccines (9) or new chemotherapeutics like the epothilones (7) have to prove their efficacy in investigational trials.

Treosulfan (L-threitol-1,4-bis(methanesulfonate), Ovastat®) serves as prodrug for a bifunctional alkylating agent which has shown efficacy in treating ovarian carcinoma and other solid non-urological tumors. The active metabolites are mono- and diepoxybutane derivates which are formed from treosulfan under physiological conditions (pH 7.4; 37°C) by nonenzymatic, spontaneous internal nucleophilic substitution (10). With a half-life of 2.2 h each, the L-diepoxybutane is established from the monoepoxide if bound to the nucleophilic centre of a biological molecule by closing a second epoxide ring after the release of one molecule methanesulfonic acid. Thus, the metabolites perform their anticancer activity by DNA alkylation of guanine bases and subsequent interstrand cross-linking (10, 11). This covalent interstrand cross-linking after preferential guanine-N7 alkylation exhibits concentration- and time-dependence (11). Cytotoxic activity predominantly.

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affects quickly proliferating cells and tissues. Treosulfan is soluble in water (12) and eliminated renally (13).

The application of treosulfan intravenously versus oral administration remains an extensively investigated topic in metastatic or recurrent ovarian carcinoma. The evaluation of the bioavailability and pharmacokinetics of both routes of drug administration have been the purpose of clinical studies (3, 5). Hilger et al. (14) and Meden et al. (15) concluded that the constant bioavailability of oral treosulfan provides a feasible long-term application of the drug until progression. Banzhaf et al. (16) treated 44 patients with ovarian cancer after complete remission following first-line therapy and suggested that long-term treosulfan therapy represents a promising option. They observed a prolonged disease-free and overall survival, remarkably in patients with advanced G3 tumors.

Treasulfan is regularly combined in first-line regimens with platinum-containing chemotherapy in advanced ovarian cancer. The evaluation of second- or third-line therapies in recurrent ovarian carcinomas frequently includes treosulfan in mono or combined regimens under study protocol conditions (17-19). Multidrug resistant solid tumors (ovarian, colorectal and renal cell cancer, ocular melanoma) have been treated in phase I dose escalating trials in combination with gemcitabine (20). Recently, treosulfan has been the subject of multiple investigations as myeloablative conditioning in a combined regimen prior to allogeneic hematopoietic stem cell transplantation in various pre-existing conditions such as hematological malignancies, lymphomas, myelodysplastic syndromes or amyloidosis, showing immunosuppressive activity against both T-cell and predominantly B-cell functions (21). In a conventional phase I trial with escalation of single dose treosulfan, hematotoxicity was described as the dose-limiting toxicity with thrombocytopenia being predominant (22). Harstrick et al. (22) considered a maximum dose of 10 mg/m² safe for administration. This dose can be escalated to more than 50 mg/m² if treosulfan is combined with hematopoietic stem cell transplantation to overcome hematotoxicity. Other observed side-effects were diarrhea, mucositis and stomatitis, skin reactions and metabolic acidosis due to the release of two moles of methanesulfonic acid during the nonenzymatic activation of one mole of treosulfan (13).

The aim of this study was to evaluate a possible cytotoxic effect of treosulfan on both hormone-sensitive and hormone-independent prostate cancer cell lines in vitro.

Materials and Methods

**Human prostate cancer cell lines.** For the experiments, the cell lines LNCaP (ATCC-LGC Promochem, Wesel, Germany), DU145 (I.A.Z., Munich, Germany) and PC3 (I.A.Z.) were used. They derive from human prostate cancer metastasis of lymph node, brain and bone, respectively and are well characterized, with different biological properties (25).

**Cell culture.** The cell lines were seeded at concentrations of 2.5x10⁴ cells/ml and cultured at 37°C in a humidified atmosphere of 5% CO₂. For LNCaP cells, MCDB (Molecular Cellular Developmental Biology)-131 medium with L-glutamine and without sodium bicarbonate supplemented with 10% fetal calf serum (FCS; Gibco/Invitrogen, Karlsruhe, Germany), 1% sodium pyruvate (Sigma, Taufkirchen, Germany), 4.5 g/l glucose (Sigma) and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gibco) was used. DU145 cells were grown in modified MEM Eagle’s medium with L-glutamine (2%; Gibco) supplemented with 10% FCS (Gibco). PC3 cells were cultivated in F-12K nutrient mixture Kaight’s modification (1x) with L-glutamine (Gibco) supplemented with 7% FCS (Gibco). All cell cultures were supplemented with the antibiotics penicillin (100 U/ml)/streptomycin (100 µg/ml; Gibco). Cell culture medium was replaced every other day. Cell growth was checked using phase-contrast microscopy.

**Treasulfan dosage.** To determine the response of the prostate cancer cell lines to treosulfan (Ovastat®; Medac GmbH, Wedel, Germany), preliminary studies were performed with cell concentrations from 1.25-5.0x10⁵ cells/ml and treosulfan concentrations in the range of 0.1-5,000 µM for a period of up to 6 days. The maximum treosulfan concentrations tested were in the range of peak concentrations detected in plasma from patients treated for ovarian cancer (13, 14). According to the data obtained for minimal and maximal cytotoxic effects compared with untreated controls, the cell lines were supplemented with different treosulfan concentrations for a period of up to 6 days. LNCaP cells were treated with 0.5-50 and 100-500 µM treosulfan, DU145 cells with 10-100 µM treosulfan and PC3 cells with 100-400 µM treosulfan. All experiments were conducted six (LNCaP) or eight (DU145, PC3) times in parallel. Control data were obtained from prostate cancer cell lines cultivated in equivalent dilutions and media without treosulfan.

**Analysis of cell proliferation and cell viability.** To determine cell proliferation and cell viability, the specific reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; Roche Applied Science, Penzberg, Germany) was used. The assay visualizes the formation of formazan from the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. The assay was performed with dilutions and media without treosulfan. The amount of resulting formazan dye directly correlates with the number of metabolically active cells. The assay was performed according to the manufacturer’s protocol. The absorbance of the dye solution was measured by a scanning microplate reader (Milenia™ Kinetic Analyzer; Molecular Devices, Sunnyvale, USA) at 450 nm with a reference wavelength of 650 nm. The data obtained were analyzed with the SOFTmax® PRO software (Version 3.0; Molecular Devices).

**Statistics.** Statistical significance was calculated with a t-test for matched pairs using JMP software (Version 3.2.6.; SAS Institute Inc., Cary, USA) for personal computers.

**Results**

**LNCaP.** Dependent on the dose of treosulfan applied, an inhibitory effect on the proliferation rate of the androgen-sensitive LNCaP tumor cells was observed. In comparison to the linear increase in cell viability of untreated controls,
the viability of LNCaP cells was statistically significantly reduced in all experiments performed for treosulfan concentrations equal to or greater than 10 μM. The cytotoxic effect of treosulfan was time-dependent. After three days, a concentration of 10 μM treosulfan showed a 2.5-fold reduction on cell viability in comparison to the controls (p<0.0001). An increase of treosulfan to 50 μM revealed a 4.9-fold inhibition (p<0.0001) of tumor cell viability (Figure 1a). The influence of 100, 250 and 500 μM of treosulfan on the viability of LNCaP cells is shown in Figure 1b. Again, a time-dependent cytotoxic effect of treosulfan was detected. Following treatment with 100 μM treosulfan, viability of LNCaP cells was 1.5-fold lower.
Table I. Effect of 100 µM treosulfan on the cell lines investigated.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treosulfan dosage</th>
<th>Inhibition of cell viability compared to controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>100 µM</td>
<td>6.8-fold</td>
<td>0.0001</td>
</tr>
<tr>
<td>DU145</td>
<td>100 µM</td>
<td>48.1-fold</td>
<td>0.0001</td>
</tr>
<tr>
<td>PC3</td>
<td>100 µM</td>
<td>6.0-fold</td>
<td>0.0001</td>
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compared with untreated controls after one day (p<0.0011) and 6.8-fold after three days (p<0.0001). Incubation with 250 µM treosulfan led to the complete destruction of the cell culture after three days, whereas doubling of the treosulfan dose to 500 µM brought about death of all cultured LNCaP cells after two days. In phase-contrast microscopy, cell damage was indicated by rounding up and detachment of cells.

**DU145.** All applied treosulfan concentrations (10-100 µM) exhibited a dose- and time-dependent cytotoxic effect on the viability of the androgen-independent cells. For all concentrations tested, the differences were statistically highly significant after one day compared to the controls (p<0.0001). A concentration of 100 µM of the drug led to a 2.1-fold decrease in cell viability after one day as compared to the untreated control cultures (p<0.0001) and to an almost complete destruction of the cultured DU145 cell line after three days (p<0.0001). Incubation with 10 µM treosulfan revealed a 1.2-fold inhibition of cell viability after one day (p<0.0001) and a 2.1-fold inhibition after three days (p<0.0001) (Figure 2).

**PC3.** The drug treosulfan showed a dose- and time-dependent cytotoxic effect on the androgen-independent PC3 cells. In comparison to the control, incubation with 100 µM treosulfan reduced the viability of PC3 cells 2.4-fold (p<0.0001) after one day and 9.1-fold (p<0.0001) after four days. Treosulfan concentrations equal to or greater than 200 µM extinguished viability of PC3 cell cultures statistically significantly (p<0.0001) after two days (Figure 3).

**Influence of 100 µM treosulfan on cell viability after three days.** DU145 cells revealed a high sensitivity for treosulfan with a viability downstage of factor 48.1, whereas the viability of LNCaP and PC3 cell lines decreased by 6.8- and 6.0-fold, respectively (Table I). Compared to the controls, the differences were statistically highly significant (p<0.0001).

**Time to extinction of cell viability dependent on treosulfan concentration.** A concentration of 500 µM treosulfan extinguished the viability of the hormone-independent DU145 cells and PC3 cells after one day and the viability of the hormone-sensitive LNCaP cells after two days. After three days, LNCaP cell cultures were eradicated by 250 µM treosulfan, whereas DU145 cell cultures were extinguished by 100 µM treosulfan and PC3 cell cultures by 200 µM treosulfan.

Concentration- and time-dependence in PC3 cells showed an almost linear trend with extinction of cell viability after one day with 500 µM, after two days with 300 µM, after three days with 200 µM and after four days with 150 µM treosulfan (Figure 4).

**Discussion**

The lack of a curative approach in metastatic prostate cancer emphasizes the inevitable urgency to define new treatment strategies. The bifunctional alkylating chemotherapeutic drug treosulfan is used for the treatment of ovarian carcinoma mainly in combined protocols. Furthermore, it is employed in myeloablative conditioning prior to allogenic hematopoietic cell transplantation in high-dose chemotherapeutic trials where a maximum tolerated dose of 47 g/m² was reported (13). The favourable toxicity profile of treosulfan and the oral availability in a metronomic regimen (24) substantiates experimental and, thus, clinical studies concerning the efficacy in metastatic disease of prostate carcinoma.

Treosulfan exhibited linear increases in plasma levels. Maximum plasma concentrations of about 4.5 mmol/l were reached in high-dose settings in vivo (13). Though incomparable to in vivo data, we observed significant toxicity of treosulfan on three well-defined prostate cancer cell lines evaluated using photospectrometric analysis which demonstrated a decrease of cell viability or death of cells when compared to untreated controls. We applied treosulfan concentrations between 0.01 and 0.5 mmol/l respectively, which is far below the maximum tolerated in vivo concentration of 4.5 mmol/l achieved by high-dose settings followed by stem cell transplantation, and of about 1.6 mmol/l observed after administration of standard doses (13). At concentrations ≥10 µM treosulfan led to significant inhibition of cell viability and cell death in all cell lines. The cell arrest was cytotoxic and irreversible.

The LNCaP cells and the low differentiated PC3 cells tolerated higher concentrations of applied treosulfan than the DU145 cells. The androgen-independent, PSA-negative DU145 prostate carcinoma cell line, derived from brain metastasis, was inhibited 48.1-fold when treated with 100 µM treosulfan whereas LNCaP and PC3 cell lines were reduced 6.8- and 6.0-fold, respectively. Increased sensitivity to other therapeutic agents of the DU145 cells was demonstrated in a study of Ullen et al. (25) who tested the impact of zoledronic acid and docetaxel on DU145 and PC3 cells and found cell numbers were reduced to 81% and 60%, respectively.
The bifunctional covalent linkage between guanine bases of cell DNA and the antitumor alkylating agents might inhibit the progress of DNA replication or transcription. Nonetheless, alkylating agents can induce cell death via several pathways. Repair enzymes may correct or enhance DNA damage, creating base deletions or misplacement of DNA single- or double-strand breaks, apurinic sites or ring openings, all resulting in insufficient DNA (26). Although covalent adduct formation on cellular DNA is the cytotoxic mechanism of all alkylating agents, their differences in the non-alkylating portions of these molecules imply the differences in pharmacokinetic biodistribution and tissue toxicity accounting for the variable potency, toxicity and disease selectivity (26). Meinhardt et al. (27) demonstrated an up-regulation of the subG1 fraction and a fourfold increase of cell death in human myeloma cell lines after two days when treated with 100 μmol/l treosulfan compared to untreated controls.

The almost linear trend with time to complete cell arrest and various concentrations of treosulfan found in each prostate cancer cell line tested demonstrates that the cytotoxic activity seems not to respond to a saturation at these concentrations. Park et al. (28) and Hartley et al. (11) demonstrated that the covalent interstrand cross-linking after preferential guanine-N7 alkylation is concentration- and time-dependent (11). The results revealed that the cytotoxic activity can further be intensified with increase of treosulfan concentration to an in vivo maximum tolerated plasma concentration. Regression analysis of the area under the curve (AUC0-∞) in relation to the treosulfan dose in vivo exhibited a linear correlation up to the MTD (maximum tolerated dose) of 47 g/m² in a pharmacokinetic analysis of Scheulen et al. (13). Hilger et al. (29) treated patients with advanced solid tumors with a single-dose intravenous 30 min infusion (8 or 10 g/m²) and measured pharmacokinetic behaviour and renal elimination of treosulfan using a validated RP-HPLC method. The terminal half-life of treosulfan found was in the range of 1.8 h. The mean urinary excretion of the parent compound was about 25% (range 5-49%) of the total dose delivered over 48 h, and about 20% was excreted during the first 6 h after administration. In dogs, the active metabolite L(+)diepoxybutane was shown to disappear from the plasma within 48 h, and about 20% was excreted during the first 6 h after intravenous infusion (30). This might be due to the lipophilic character and rapid reaction with nuclear sites (29). Therefore, measuring treosulfan plasma and urine concentration remains the main target of pharmacokinetic studies in search of Cmax (maximal plasma concentration), AUC0-∞ and bioavailability for novel treatment protocols.

Considering its favourable toxicity profile, we suggest further investigations to evaluate treosulfan in the treatment of prostate carcinoma. Due to a lack in cross-resistance reported between treosulfan and other cytotoxic agents, treosulfan might be promising in first- and second-line chemotherapy regimens in prostate cancer.

References


